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# Refined Characterization of the Expression and Stability of the *SMN* Gene Products

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**Spinal muscular atrophy (SMA) is characterized by degeneration of lower motor neurons and caused by mutations of the *SMN1* gene. *SMN1* is duplicated in a homologous gene called *SMN2*, which remains present in patients. SMN has an essential role in RNA metabolism, but its role in SMA pathogenesis remains unknown. Previous studies suggested that in neurons the protein lacking the C terminus ($SMN^{\Delta 7}$), the major product of the *SMN2* gene, had a dominant-negative effect. We generated antibodies specific to $SMN^{FL}$ or $SMN^{\Delta 7}$. In transfected cells, the stability of the $SMN^{\Delta 7}$ protein was regulated in a cell-dependent manner. Importantly, whatever the human tissues examined, $SMN^{\Delta 7}$ protein was undetectable because of the instability of the protein, thus excluding a dominant effect of $SMN^{\Delta 7}$ in SMA. A similar decreased level of $SMN^{FL}$ was observed in brain and spinal cord samples from human SMA, suggesting that $SMN^{FL}$ may have specific targets in motor neurons. Moreover, these data indicate that the vulnerability of motor neurons cannot simply be ascribed to the differential expression or a more dramatic reduction of $SMN^{FL}$ in spinal cord when compared with brain tissue. Improving the stability of $SMN^{\Delta 7}$ protein might be envisaged as a new therapeutic strategy in SMA.** ***(Am J Pathol 2007, 171:1269–1280; DOI: 10.2353/ajpath.2007.070399)***

Spinal muscular atrophies (SMAs) (Online Mendelian Inheritance of Man nos. 271150, 253550, 253400, 253300; *http://www.ncbi.nlm.nih.gov/omim/*) are characterized by the degeneration of lower motor neurons, leading to progressive limb and trunk paralysis associated with muscular atrophy. SMA is a frequent recessive autosomal disorder caused by mutations of the survival of motor neuron gene (*SMN1*).[1] *SMN1* is duplicated as a highly homologous gene, called *SMN2*, and both genes are transcribed.[1] The *SMN2* gene is present in all patients but is not able to compensate for *SMN1* gene defects. At the genomic level, the gene dosage effect found in type I SMA, but not in type III SMA, has suggested that type I is caused by deletion of *SMN1*, whereas type III is associated with a conversion event of *SMN1* into *SMN2*, leading to an increased number of *SMN2* genes.[1,2] This is in agreement with the tight inverted correlation between the amount of protein encoded by the *SMN2* gene and the clinical severity of human SMA disease.[3,4]

Five nucleotides distinguish *SMN2* from *SMN1* without altering the amino acid sequence.[1] The critical difference between these two genes is a cytosine (C) to thymine (T) transition in exon 7 of *SMN2*, which is responsible for the alternative splicing of exon 7 of SMN2 transcripts.[5] Full-length transcripts are almost exclusively produced by the *SMN1* gene, whereas the predominant form encoded by *SMN2* lacks exon 7 ($SMN^{\Delta 7}$).[1,5] Full-length transcript ($SMN^{FL}$) is also encoded by *SMN2* and translated into functional protein. However, it is a minor form, much less abundant than the full-length *SMN1* gene product. The truncated transcript lacking exon 7 encodes a putative shorter protein in which the last 16 residues of $SMN^{FL}$ are replaced by four residues (EMLA) encoded by exon 8 ($SMN^{\Delta 7}$). Using expression vectors, *in vitro* experiments demonstrated that $SMN^{\Delta 7}$ oligomerizes less efficiently than the full-length form and that overexpressed $SMN^{\Delta 7}$ was unstable in a nonneuronal immortalized cell line.[6,7]

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However, in these studies, the stability of $SMN^{\Delta 7}$ in humans was not elucidated.

SMN is a ubiquitously expressed protein of 294 amino acids, with a molecular mass of 38 kd. SMN forms a large multiprotein complex of ~1 Md, both in the cytoplasm and in the nucleus, where it is concentrated in a structure called gem (for gemini of coiled bodies).[8,9] The identification of SMN-interacting proteins of known function in nonneuronal cell lines strongly supports the view that SMN is involved in, and facilitates, cytoplasmic assembly of snRNP into the spliceosome, a large RNA-protein complex that catalyzes the splicing reaction.[10,11] In the nucleus, SMN appears to be directly involved in pre-mRNA splicing, transcription, and metabolism of ribosomal RNA.[11,12] More recently, it has been suggested that SMN might have an additional function in neurons related to RNA trafficking. SMN binds heterogeneous nuclear ribonucleoprotein-R (hnRNP-R), an mRNA-binding protein that may associate with β-actin mRNA *in vitro*, suggesting a role for SMN in the assembly and/or transport of β-actin mRNP complexes into growth cones.[13] Moreover, SMN was localized in granules and was transported down axons of cultured neurons.[14] Together, these data suggested that SMN plays a role in the metabolism of RNA in the nucleus and/or in the transport of some transcripts in axons.

Several hypotheses have been raised to explain the higher vulnerability of motor neurons in response to the *SMN1* mutation. *In vivo*, the link between RNA metabolism and SMA pathogenesis has been suggested. However, one report did not find a single abnormal splicing pattern in mice carrying a heterozygous deletion of the *Smn* gene.[15] Expression profiles of 8400 genes in mouse skeletal muscle and spinal cord expressing $SMN^{\Delta 7}$ RNA only revealed an early, and specific, up-regulation of genes involved in pre-mRNA splicing, ribosomal RNA processing, and RNA decay.[16] The observed changes could represent an adaptive response of the RNA-processing machinery because of the lack of a component normally involved in the process. These results thus provide indirect evidence for a role of SMN in RNA metabolism *in vivo*. However, overexpression of orthologous RNA metabolism-related genes, specific to the SMN defect, was not detected in skeletal muscle or spinal cord of human SMA samples, at either fetal or postnatal stages.[16] The difference between mouse and human data could be attributable to the reduction, but not the absence, of $SMN^{FL}$ in human SMA tissues or to a defect in the metabolism of some RNA subclasses involved in the structural specificities of motor neurons. In agreement with this hypothesis, a correlation was found between the capacity for snRNP assembly and SMN protein level in SMA type I patients.[17] The link between RNA metabolism and SMA pathogenesis has been also strongly suggested by the ability of injected U snRNPs to prevent motor neuron degeneration in SMN-deficient zebrafish embryos.[18] Surprisingly, more recent data have shown that the function of SMN in motor axon outgrowth of zebrafish might be dissociated from snRNP function, suggesting that the motor axon defect observed in zebrafish mutated for SMN is unlinked to snRNP biosynthesis.[19] The developmental defect of motor axon growth has, however, not been reported to date in human SMA autopsy materials.

*In vitro*, overexpression of $SMN^{\Delta 7}$ seems to be pro-apoptotic.[20,21] In addition, in chick forebrain neurons, $SMN^{\Delta 7}$ was unable to be transported, and overexpression of $SMN^{\Delta 7}$ resulted in shorter axons associated with the retention of $SMN^{\Delta 7}$ in the nucleus.[14] These data suggested that, in addition to the deleterious effect of a reduced dose of $SMN^{FL}$, $SMN^{\Delta 7}$ might have a dominant-negative effect on neurons.[14,20,21] However, these data were not confirmed using a different culture system.[22]

Motor axons can reach more than 1 m long, making motor neurons one of the largest cells in the body in terms of both volume and surface area. Such large cellular units require molecular machinery able to produce and regulate molecules, including proteins and RNA, from the cell body to the neuromuscular junction, through motor axons. Neurons express high numbers of genes with respect to other cell types, and alternative splicing as well as the stability of mRNAs are important posttranscriptional mechanisms necessary to create and regulate protein diversity.[23]

In summary, several hypotheses have been raised to explain the vulnerability of motor neurons in SMA: i) a dominant effect of $SMN^{\Delta 7}$ in motor neurons; ii) a more dramatic reduction of SMN in spinal cord compared with other tissues or a higher demand of SMN in motor neurons, attributable to their large size; iii) a role for SMN in the metabolism of a subclass of RNA specific to motor neurons; or iv) an additional function of SMN in these cells, unrelated to snRNP biogenesis. Here, we generated antibodies specific to $SMN^{\Delta 7}$ and $SMN^{FL}$ to clearly determine the presence or absence of the SMN proteins in normal and disease states. We show that the $SMN^{\Delta 7}$ protein is undetectable in human lymphoblastoid cell lines or tissues, including neuronal tissues. In addition, we show that the stability of the $SMN^{\Delta 7}$ protein depends on the cell type. The SMN protein detected in SMA samples is translated only from the full-length SMN2 transcript, which results in the dramatic decreased level of SMN in all of the early fetal SMA tissues examined. In addition to therapeutic strategies aimed at either up-regulating *SMN2* gene expression or at limiting exon 7 skipping, improving the stability of the $SMN^{\Delta 7}$ protein could represent a new attractive therapeutic strategy in SMA.

## Materials and Methods

### Plasmids

The human $SMN^{FL}$ and $SMN^{\Delta 7}$ cDNA were amplified by polymerase chain reaction (PCR) and cloned in-frame to the 3′ end of *GFP*, at the *Mlu*I and *Xma*I restriction sites of the pCX-LGN plasmid (called *GFP* plasmid in the text), derived from pCX-EGFP (gift from IGBMC, Strasbourg, France). The *GFP*, *GFP-SMN*$^{FL}$, and *GFP-SMN*$^{\Delta 7}$ plasmids were purified using the EndoFree plasmid maxi kit (Qiagen S.A., Courtaboeuf, France), and both plasmids were checked by sequencing both strands.

## Generation of Antibodies

Rabbit $SMN^{FL}$- or $SMN^{\Delta 7}$-specific antibodies were generated against two synthetic peptides chosen in the human amino acid sequence encoded by exon 7 (peptide hsmnEx7, GFRQNQKEGRCSHSLN) or exon 6 fused to 8 (peptide hsmnEx8, GYYMEMLA), respectively. These peptides were conjugated to KLH and injected into rabbits, and antisera hSMNex7-5381 and hSMNex8-5699 were collected and purified on an affinity column (Invitrogen, Paisley, UK).

## Human Cell Lines and Tissues

### Lymphoblastoid Cell Lines

Three control cell lines harboring two *SMN1* genes and 0 (10-253), one (10-252), or two (11-771) *SMN2* genes, three type I SMA patients (10-235, 10-237, and 10-238) with two *SMN2* genes, and two type III SMA patients (10-240 and 10-241) with four *SMN2* genes were selected for Western blot analysis. SMA patients were born to consanguineous parents. Cells were maintained in RPMI 1640 medium, supplemented with 20% fetal bovine serum, 1% glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen).

### Human Fibroblasts

Primary cultures of fibroblasts were established from skin biopsies of two type I, two type II, and two type III SMA patients. Type I patients had two *SMN2* genes (SMAI-1 and SMAI-2), type II patients had three *SMN2* genes (SMAII-1 and SMAII-2), a type III patient (SMAIII-1) had three *SMN2* genes, and the other one (SMAIII-2) had four *SMN2* genes. Control fibroblasts had two *SMN1* genes and two *SMN2* genes (Cont-1, Cont-2). Fibroblasts were analyzed below 10 *in vitro* passages and maintained in Dulbecco's modified Eagle's medium with 4.5% glucose, supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen) as previously described.[24]

### Fetal Tissues

Two fetuses predicted to have type I SMA (SMA-I-3 and SMA-I-4) and one type II SMA (SMA-II-3), as well as three fetuses affected with diseases unrelated to SMA (Cont 3 to 5), were studied. The fetuses were at 11 to 18 weeks of gestation. Samples were collected in the framework of prenatal diagnosis, following the informed consent of parents in Necker-Enfants Malades (Paris, France) or Antoine Beclère (Clamart, France) hospitals. The number of *SMN1* and *SMN2* genes was determined as previously described.[25] Fetal tissues used for controls and SMA fetuses were distinct from those used for lymphoblastoid cell lines or for fibroblast analyses. SMA patients and fetuses carried a homozygous deletion of *SMN1* exon 7 (data not shown). Informed consent was obtained from all patients and controls or from their parents.

## Cell Cultures and Transfection Experiments

### HeLa Cell Transfections

HeLa cells were maintained at 37°C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen). Cells were transfected using 1 $\mu$g of DNA (*GFP*, *GFP-SMN*$^{FL}$, or *GFP-SMN*$^{\Delta 7}$ plasmids) and 2 $\mu$l of Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours after transfection.

### Primary Cultures of Mouse Cortical Neurons

Primary cultures of cortical neurons were prepared from 14.5-day postcoitum C57/BL6 wild-type mouse embryos. The cortices of embryos were dissected in $Mg^{2+}$, $Ca^{2+}$-free Hanks' balanced salt solution, trypsinized, dissociated, and then transfected using a Nucleofector apparatus (Amaxa Biosystems, Cologne, Germany) with 2 $\mu$g of DNA (*GFP*, *GFP-SMN*$^{FL}$, or *GFP-SMN*$^{\Delta 7}$ plasmids) for 5 million cells. Transfected neurons were plated on poly-D-ornithine (5 mg/ml)- and laminin (2 mg/ml)-coated dishes, at a density of $9.3 \times 10^4$ cells/cm$^2$ in Neurobasal medium (Invitrogen) plus B27 supplement. Cells were maintained for 6 days at 37°C in 5% $CO_2$. Three independent transfection experiments were performed.

One, three, or six days after transfection, the same fields were analyzed by phase contrast or fluorescence using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY). Cell survival was estimated from the mean number of nuclei on five same phase contrast fields 1, 3, and 6 days after transfection. The total neurite length, relative to the number of nuclei per field, was analyzed 1, 3, and 6 days after plating from the same five fields by using the stereological method, as previously described.[26]

Cultures were fixed after 1 day with 4% paraformaldehyde for 30 minutes at 4°C, permeabilized with 0.5% Triton X-100, incubated with 5% nonfat dry milk and 0.05% Tween 20 for 1 hour at room temperature, and then incubated in anti-20S proteasome antibody (1:500; Biomol International, Exeter, UK) overnight at 4°C. After rinsing, the cultures were incubated with anti-rabbit IgG conjugated to Alexa 594 (1:500; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cultures were then mounted with Vectashield-DAPI (Vector Laboratories, Burlingame, CA) and observed under a Zeiss Axioplan 2 fluorescence microscope.

### Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts were prepared from 14.5-day postcoitum C57/BL6 wild-type embryos following standard procedures and cultured in Dulbecco's modified Eagle's medium and 10% fetal bovine serum. Mouse embryonic fibroblasts were transfected using Nucleofector apparatus (Amaxa Biosystems) with 5 $\mu$g of DNA (*GFP*, *GFP-SMN*$^{FL}$, or *GFP-SMN*$^{\Delta 7}$ plasmids) for 12.5 million cells.

### *Protein Stability Assay*

Primary cultures of mouse cortical neurons or embryonic fibroblasts were transfected with *GFP-SMN$^{FL}$* or *GFP-SMN$^{\Delta 7}$* as described above. Twenty-four hours after transfection, cells were treated with 10 $\mu$g/ml cycloheximide (Sigma, St. Louis, MO) to inhibit protein synthesis. The cycloheximide-treated cells were harvested at different time points (0, 1, 3, and 6 hours after treatment) and processed for immunoblotting with anti-GFP. Anti-tubulin antibodies were used as internal control for loading.

### *Immunoblotting Experiments*

Total proteins were extracted from HeLa cells or primary cultures of mouse cortical neurons in a buffer containing 25 mmol/L sodium phosphate, pH 7.2, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1% sodium dodecyl sulfate supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and boiled for 10 minutes. For lymphoblastoid cells, proteins were extracted using 100 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 100 mmol/L NaCl, 5 mmol/L EDTA, 0.5 mmol/L $MgCl_2$, 1 mmol/L $\beta$-mercaptoethanol, and 1 mmol/L phenylmethyl sulfonyl fluoride supplemented with protease inhibitor cocktail (Roche). Human fibroblast cells were resuspended in a buffer containing 62.5 mmol/L Tris-HCl, pH 6.8, 5 mmol/L EDTA, and 10% sodium dodecyl sulfate, supplemented with protease inhibitor cocktail (Roche).

Samples were passed through a 1-ml syringe with a 27-gauge needle at least 20 to 25 times. Tissues from fetuses were rapidly frozen and crushed in liquid nitrogen using a mortar and pestle. Pulverized tissue samples were transferred into 5 vol of a buffer containing 25 mmol/L sodium phosphate (pH 7.2), 5 mmol/L EDTA, and 1% sodium dodecyl sulfate supplemented with protease inhibitor cocktail (Roche), and boiled for 5 minutes. Protein samples were mixed with an equal volume of 2× Laemmli buffer, electrophoresed, and then transferred.

For SMN, 20S proteasome, and tubulin immunodetection, anti-SMNtl (1:2000 to 1:10,000; BD Transduction Laboratories, San Jose, CA), anti-20S proteasome (1:10,000; Biomol International), and anti-tubulin monoclonal antibodies (1:20,000; Sigma) were used. After washes in phosphate-buffered saline and 0.05% Tween 20, membranes were incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 to 1:20,000; Jackson ImmunoResearch, West Grove, PA), and the immune complexes were revealed using chemiluminescent detection reagents (Pierce, Rockford, IL). For GFP, anti-GFP polyclonal antibody (1:2000 to 1:8000; Molecular Probes) was used and revealed using anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Jackson ImmunoResearch). For the detection of SMN$^{FL}$ and SMN$^{\Delta 7}$ proteins, hSMNex7-5381 and hSMNex8-5699 purified antisera generated in this study were diluted at 1:100 and revealed by anti-rabbit IgG conjugated to horseradish peroxidase (1:20,000; Jackson ImmunoResearch).

### *DNA and RNA Analysis*

Total DNA was extracted from primary culture of cortical neurons transfected with plasmids encoding GFP, GFP-SMN$^{FL}$, or GFP-SMN$^{\Delta 7}$ and amplified with primers *R-GFP* (5′-TCTTCAAGTCCGCCATGCCC-3′) and *F-GFP* (5′-TGTACTCCAGCTTGTGCCCC-3′). Interleukin 2 was co-amplified as an internal control using primers *IMR0042* (5′-CTAGGCCACAGAATTGAAAGATCT-3′) and *IMR0043* (5′-GTAGTTGGAAATTCTAGCATCATCC-3′).

Total RNA was extracted from lymphoblastoid cells or primary culture of cortical neurons using the TRIzol procedure (Invitrogen). First-strand cDNA synthesis was performed by using oligo(dT) primers. Negative controls without reverse transcriptase (RT) were also performed. PCR amplification analysis of single-strand cDNA was performed using primers flanking *SMN* exon 7 (*541C544*, 5′-GAGAACTCCAGGTCTCCTGGA-3′ and *541Ci993*, 5′-CGCTTCACATTCCAGATCTGT-3′) and amplified SMN transcripts containing (450 bp) or lacking exon 7 (396 bp). Transcript analysis of cortical neurons transfected by plasmids encoding GFP, GFP-SMN$^{FL}$, or GFP-SMN$^{\Delta 7}$ was performed using primers *R-GFP* (5′-TCTTCAAGTCCGCCATGCCC-3′) and *F-GFP* (5′-TGTACTCCAGCTTGTGCCCC-3′). As internal control for RT-PCR amplification analysis, *Aldolase A* or *$\beta$-actin* cDNA were co-amplified using primers *Aldo 1* (5′-TAAGAAGGATGGAGCCGACTTTG-3′) and *Aldo 600* (5′-GCGAGGCTGTTGGCCAGGGCGCG-3′) or *$\beta$AHU1* (5′-CCAACCGCGAGAAGATGACCCAG-3′) and *$\beta$AHU2* (5′-GGAAGAGTGCCTCAGGGCAGCG-3′), respectively. RT-PCR products were separated by agarose gel electrophoresis and labeled with ethidium bromide.

## *Results*

### *Generation of New Polyclonal Antibodies Specific to SMN$^{FL}$ and SMN$^{\Delta 7}$*

Peptides hSMNex7 and hSMNex8 were chosen from the human amino acid sequence encoded by *SMN* exon 7 or exon 6 fused to exon 8, which are specific to the SMN$^{FL}$ or SMN$^{\Delta 7}$ isoforms, respectively. The peptides were synthesized, coupled with KLH and injected into rabbits. The best immune response was obtained with antiserum hSMNex7-5381 (corresponding to peptide hSMNex7) and hSMNex8-5699 (corresponding to peptide hSMNex8) as monitored by an enzyme-linked immunosorbent assay (data not shown). Antisera were purified by immunoaffinity beads bearing the corresponding peptides. The human *SMN$^{FL}$* and *SMN$^{\Delta 7}$* cDNA were fused to the 3′ end of GFP (called *GFP-SMN$^{FL}$* and *GFP-SMN$^{\Delta 7}$*, respectively) and cloned into the expression plasmid pCX-LGN. Sequence analysis of cloned cDNA showed, as expected, a nucleotide sequence identical to human wild-type *SMN$^{FL}$* or *SMN$^{\Delta 7}$* cDNA (data not shown). Transfection experiments of HeLa cells with these plasmids, or with GFP alone, were performed. Proteins were extracted from transfected and nontransfected cells and analyzed by immunoblotting experiments using antisera hSMNex7-

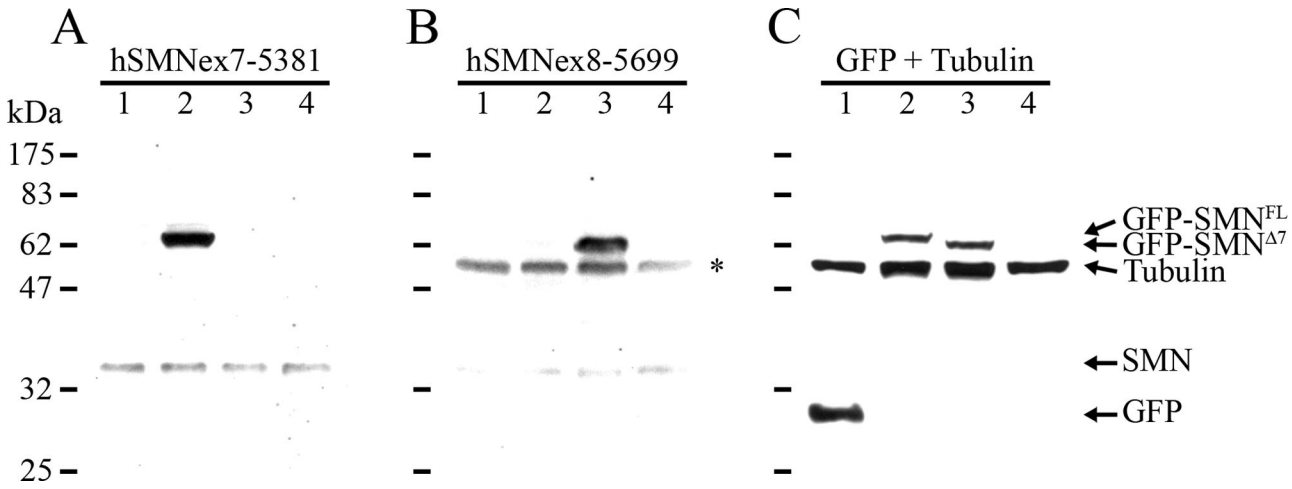


**Figure 1.** Characterization of new polyclonal antibodies directed against human $SMN^{FL}$ and $SMN^{\Delta 7}$ proteins. Immunoblot analysis of proteins extracted from HeLa cells transfected with a plasmid expressing GFP (**lane 1**), GFP-$SMN^{FL}$ (**lane 2**), GFP-$SMN^{\Delta 7}$ (**lane 3**), or from nontransfected HeLa cells (**lane 4**). The membranes were incubated with hSMNex7-5381 (**A**), hSMNex8-5699 (**B**) antisera, or with GFP and tubulin antibodies (**C**). An **asterisk** indicates a nonspecific band (see comment on Figure 7).

5381, hSMNex8-5699, or antibody against GFP (Figure 1). hSMNex7-5381 antiserum detected both the recombinant GFP-$SMN^{FL}$ (65 kd) and endogenous proteins (38 kd) but not, as expected, GFP-$SMN^{\Delta 7}$. The hSMNex8-5699 antiserum detected the recombinant GFP-$SMN^{\Delta 7}$ (64 kd) but not the recombinant $SMN^{FL}$ proteins (Figure 1). A faint band similar in size to that of the SMN endogenous band was inconsistently detected in HeLa cells (Figures 1, 7, and 8). No cross-reactivity of hSMNex7-5381 and hSMNex8-5699 antisera against $SMN^{\Delta 7}$ and $SMN^{FL}$ recombinant proteins, respectively, was detected.

## *Overexpression of $SMN^{FL}$ or $SMN^{\Delta 7}$ Reveals the Dramatic Instability of $SMN^{\Delta 7}$ Protein in Neurons but Not in Other Cell Types in Culture*

Previous reports have shown that overexpression of $SMN^{\Delta 7}$ resulted in shorter axons associated with nuclear retention of the $SMN^{\Delta 7}$ protein or in cell death. These data suggested a dominant-negative effect of $SMN^{\Delta 7}$.[14,20,21] To determine whether this effect was observed in mammalian cells, plasmids encoding GFP-$SMN^{FL}$, GFP-$SMN^{\Delta 7}$, or GFP only were transfected into various cell types including HeLa cells, cortical neurons, or fibroblasts from mouse embryos.

In HeLa cells, transfection experiments using these plasmids, or with GFP alone, led to the overexpression of both fused proteins, as determined by immunoblotting experiments using antisera hSMNex7-5381, hSMNex8-5699, or antibody against GFP only (Figure 1). Microscopic examination of transfected cells revealed the presence of $SMN^{FL}$ or $SMN^{\Delta 7}$ aggregates in both the cytoplasm and the nucleus (Figure 2). To determine whether similar findings were observed in mammalian neuronal cells, cortical neurons were collected from 14.5-day postcoitum mouse embryos and transfected with *GFP-$SMN^{FL}$*, *GFP-$SMN^{\Delta 7}$*, or *GFP* constructs, plated, and observed for 6 successive days. The density of neurites was evaluated in neurons 1, 3, and 6 days after cell plating, by examining the same fields in phase contrast, and did not reveal any difference whatever with the transfected construct (Figure 3D). These data indicate that overexpression of $SMN^{FL}$ or $SMN^{\Delta 7}$ did not have a deleterious effect on neurite outgrowth of mouse neurons in culture when compared with GFP alone. Microscopic examination of neurons transfected with *GFP-$SMN^{FL}$* revealed aggregates of SMN in both the nucleus and the cytoplasm, including the neurites (Figure 2 and see Supplemental Figure 1 at *http://ajp.amjpathol.org*). Surprisingly, however, in cortical neurons transfected with *GFP-$SMN^{\Delta 7}$*, no or very weak fluorescent signal was observed even after immunolabeling of GFP (Figure 2 and see Supplemental Figure 2 at *http://ajp.amjpathol.org*; and data not shown).

To clarify the reasons for such a dramatic reduction in GFP-$SMN^{\Delta 7}$ protein in neurons, semiquantitative analysis of DNA, RNA, and protein encoding GFP-$SMN^{FL}$ or GFP-$SMN^{\Delta 7}$ was performed 1 day after the transfection of cortical neurons (Figure 3). Despite similar levels of plasmid DNA, reduced levels of GFP-$SMN^{\Delta 7}$ RNA (48%) and protein (81% reduction, $P = 0.001$) were detected when compared with the GFP-$SMN^{FL}$ RNA or protein levels and internal controls (Figure 3). To determine whether these data might result from cell death, the survival of neurons transfected with *GFP-$SMN^{\Delta 7}$*, *GFP-$SMN^{FL}$*, or *GFP* alone was analyzed. The survival of neurons was similar regardless of the plasmid used or the day of examination (from days 1 to 6, Figure 3). These data indicate a marked instability of GFP-$SMN^{\Delta 7}$ RNA and protein in neurons, but not in HeLa cells, when compared with either GFP-$SMN^{FL}$ or GFP (Figures 1 and 3).

To determine whether this instability is found in other cell types, the same constructs were transfected into a primary culture of fibroblasts from wild-type mouse embryos (Figure 2). Interestingly, fibroblasts transfected with *GFP-$SMN^{FL}$* display numerous aggregates mainly in the cytoplasm, with very few in the nucleus. In contrast, numerous aggregates were detected in both the nucleus

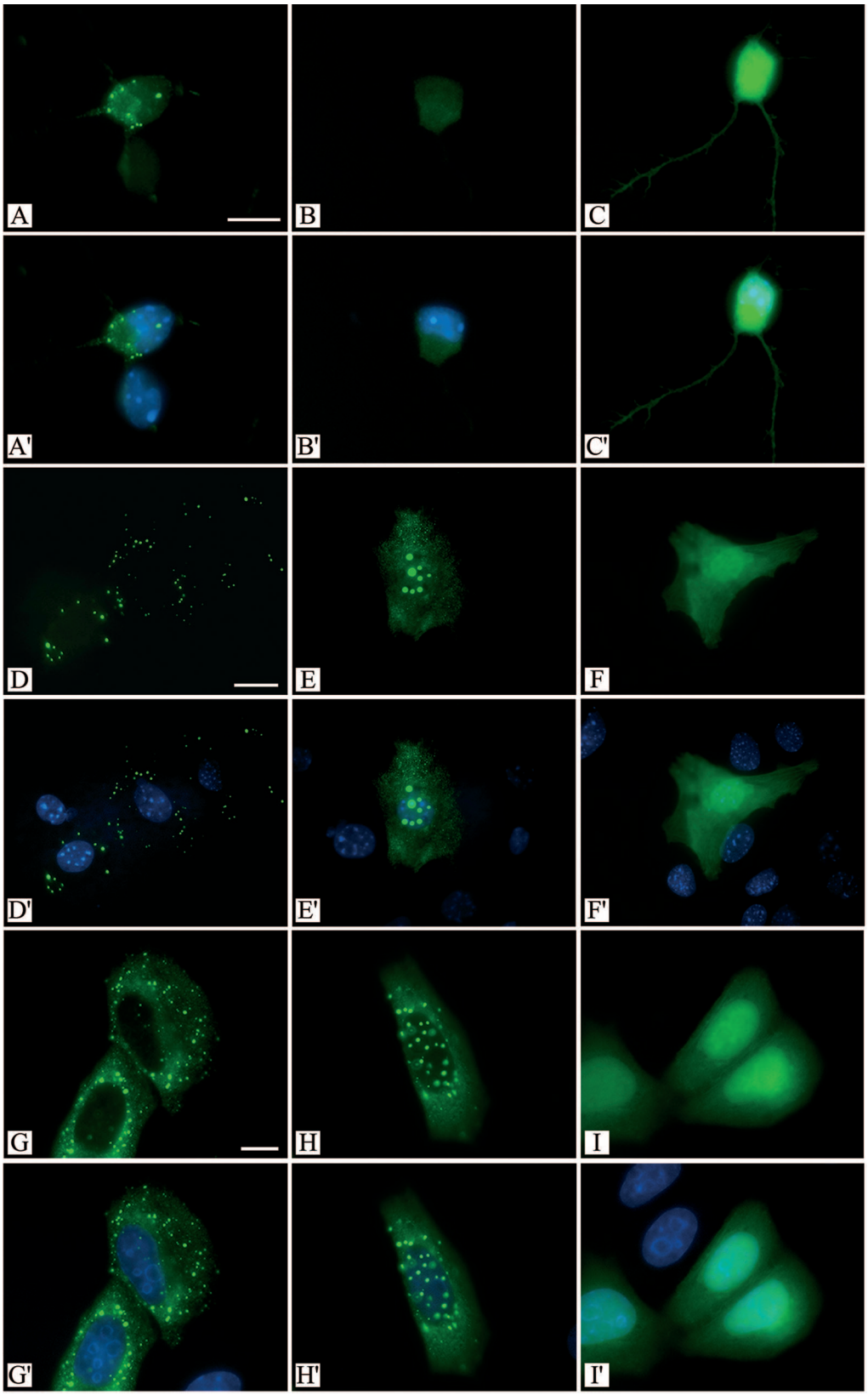


**Figure 2.** Expression and subcellular localization of $SMN^{FL}$ and $SMN^{\Delta 7}$ in various mammalian cell types. Primary cultures of mouse cortical neurons (**A–C′**), mouse fibroblasts (**D–F′**), and HeLa cells (**G–I′**) were transfected with a plasmid expressing GFP-$SMN^{FL}$ (**A**, **D**, and **G**), GFP-$SMN^{\Delta 7}$ (**B**, **E**, and **H**), or GFP (**C**, **F**, and **I**). GFP-$SMN^{FL}$ is concentrated as aggregates in the cytoplasm and in the nucleus of neurons (**A**), fibroblasts (**D**), and HeLa cells (**G**). GFP-$SMN^{\Delta 7}$ is localized in both the nucleus and cytoplasm of fibroblasts (**E**) and HeLa cells (**H**). **B:** Despite normal growth and survival of GFP-$SMN^{\Delta 7}$-transfected neurons, only a very weak signal can be seen in the cytoplasm. Nuclei are stained with 4,6-diamidino-2-phenylindole. **A′–C′**, **D′–F′**, and **G′–I′** are merged images. Scale bars: 10 $\mu$m (**A–C′**, **G–I′**); 20 $\mu$m (**D–F′**).

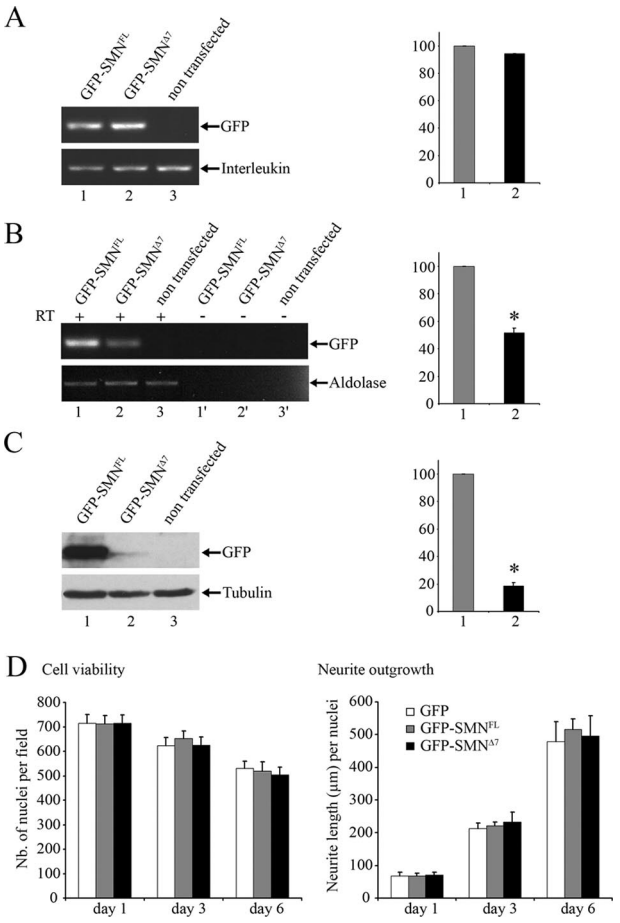


**Figure 3.** Characterization of primary cultures of cortical neurons transfected with a plasmid expressing GFP-SMN$^{FL}$ (**lane 1**), GFP-SMN$^{\Delta 7}$ (**lane 2**), and nontransfected control (**lane 3**). Quantification of plasmid DNA through PCR amplification of *GFP* sequence (**A**), RNA using RT-PCR amplification (**B**), or GFP fusion protein by immunoblot experiment (**C**) 1 day after plating. Although a similar amount of transfected plasmid was used, a marked decrease in RNA (48%) and protein (81%) encoding GFP-SMN$^{\Delta 7}$ was observed when compared with GFP-SMN$^{FL}$. Relative amounts of plasmid DNA, RNA encoding GFP, and GFP protein were evaluated by determining the ratio of transgene to internal control (interleukin for DNA, aldolase for RNA, and tubulin for protein analyses). For transcript analysis, PCR amplification was performed with (+) or without (−) reverse transcriptase as a negative control. **D:** Cell viability was estimated from the mean number of nuclei on five same-phase contrast fields at 1, 3, and 6 days of culture. The neurite outgrowth was measured 1, 3, and 6 days after plating of transfected cortical neurons by a stereological procedure on five same-phase contrast fields. No statistically significant difference was observed whatever the transfected plasmids or the day of examination. Three independent experiments were performed. Bar means SE.

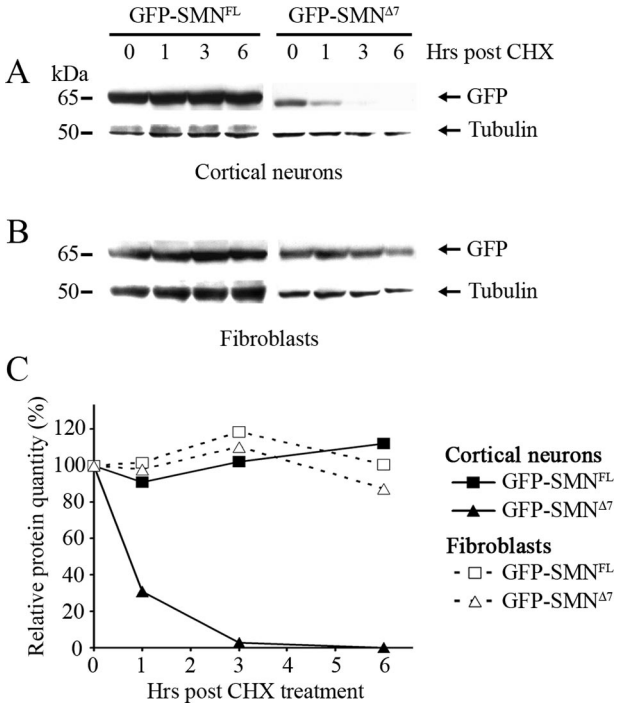


**Figure 4.** SMN protein stability assay in neurons and fibroblasts. Primary cultures of mouse cortical neurons (**A**) and embryonic fibroblasts (**B**) were transfected with a plasmid expressing GFP-SMN$^{FL}$ or GFP-SMN$^{\Delta 7}$. Cells were treated with cycloheximide (CHX) 24 hours after transfection, and proteins were harvested at indicated time points (0, 1, 3, and 6 hours after CHX treatment). Similar amounts of total protein extracts were loaded and analyzed by Western blot using anti-GFP and anti-tubulin antibodies. **C:** Levels of GFP-SMN$^{FL}$, GFP-SMN$^{\Delta 7}$, and tubulin proteins were evaluated by densitometric analysis. Each point corresponds to the ratio of the fused protein: tubulin relative to that at time point 0.

and the cytoplasm of fibroblasts transfected with the *GFP-SMN*$^{\Delta 7}$ construct. These data indicate that the stability of SMN$^{\Delta 7}$ protein depends on the cell type; it is much more unstable in neurons than in the other cell types.

To compare further the protein stability of GFP-SMN$^{FL}$ and GFP-SMN$^{\Delta 7}$ proteins in different cell types, primary cultures of mouse cortical neurons and fibroblasts were transfected with *GFP-SMN*$^{FL}$ and *GFP-SMN*$^{\Delta 7}$ plasmids and then treated with cycloheximide to inhibit protein synthesis (Figure 4). The levels of fused proteins have been analyzed 0, 1, 3, and 6 hours after treatment and compared with that of tubulin used as internal control. A dramatic reduction of the relative amount of GFP-SMN$^{\Delta 7}$ was observed in neurons when compared with the full-length protein (GFP-SMN$^{FL}$). Indeed, the GFP-SMN$^{\Delta 7}$ protein disappeared almost completely 3 hours after treatment contrasting with the stability of the GFP-SMN$^{FL}$ (Figure 4). In fibroblasts, however, the stability of GFP-SMN$^{\Delta 7}$ and GFP-SMN$^{FL}$ was similar (Figure 4). These data indicate that the stability of the SMN$^{\Delta 7}$ protein is regulated in a cell-dependent manner.

To determine whether the instability of the SMN$^{\Delta 7}$ protein is linked to the activation of the ubiquitin proteasome pathway, immunolabeling and Western blot analysis of the 20S proteasome was performed in HeLa and cortical neurons transfected with *GFP-SMN*$^{\Delta 7}$or *GFP-SMN*$^{FL}$. No change in the expression or subcellular localization of the 20S proteasome was observed under any of the conditions analyzed (Figure 5 and see Supplemental Figure 3 at *http://ajp.amjpathol.org*).

## *Tight Correlation between SMN$^{FL}$ and SMN2 Gene Number and the Lack of SMN$^{\Delta 7}$ Protein in Human SMA Cell Lines*

Previous studies have shown a correlation between SMN protein level and the severity of the disease.[3,4,24] However, without specific antibodies to SMN$^{FL}$ or SMN$^{\Delta 7}$, it was not possible to determine whether the detected protein corresponded to SMN$^{FL}$, SMN$^{\Delta 7}$, or both protein

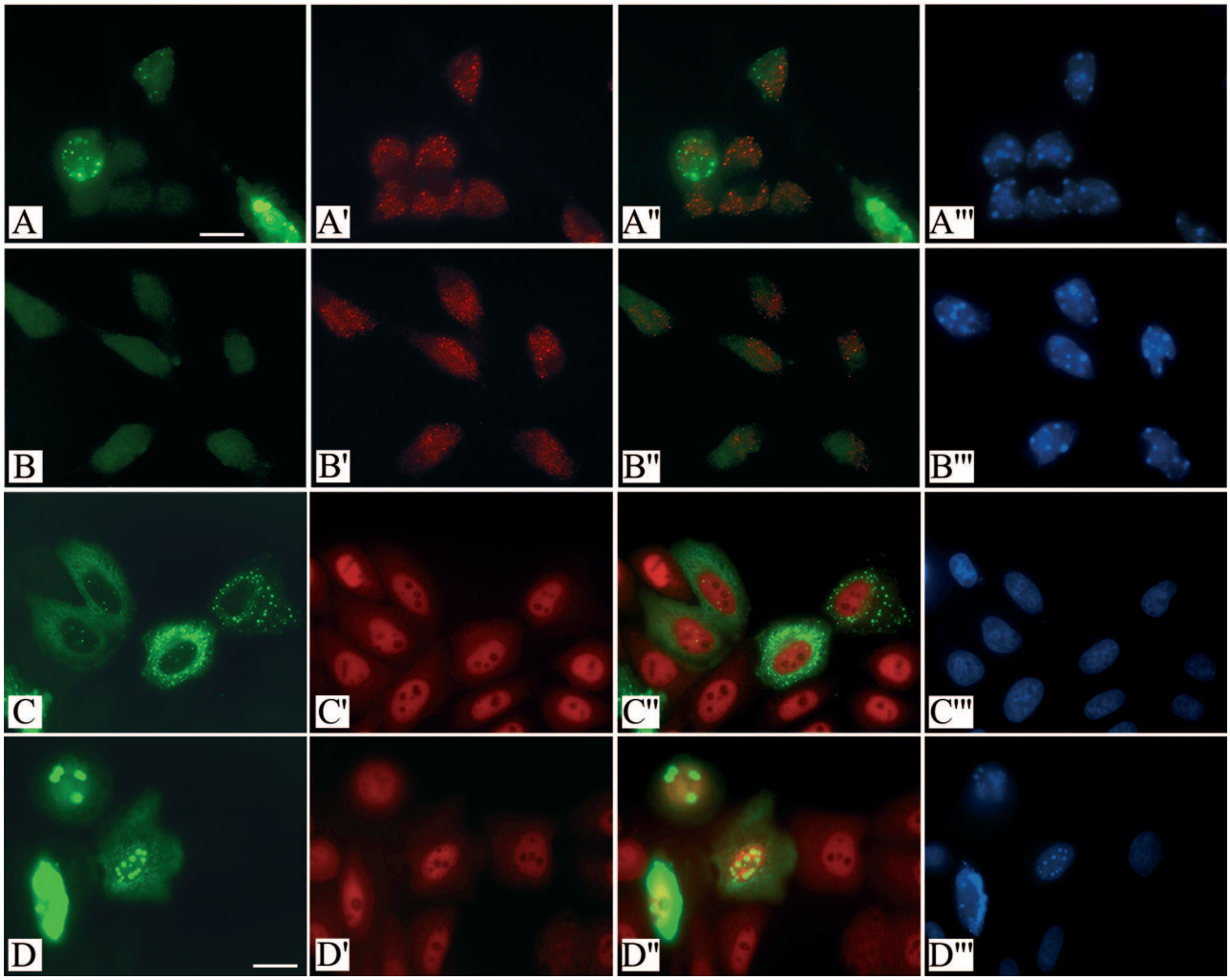


**Figure 5.** Immunolabeling experiment of the 20S proteasome in HeLa and cortical neurons. Primary cultures of mouse cortical neurons (**A–B′′′**) or HeLa cells (**C–D′′′**) were transfected with a plasmid expressing GFP-SMN$^{FL}$ (**A–A′′′** and **C–C′′′**) or GFP-SMN$^{\Delta 7}$ (**B–B′′′** and **D–D′′′**). The 20S proteasome is labeled in red (**A′–D′**) and GFP fluorescence is shown in green (**A–D**). Nuclei were stained with 4,6-diamidino-2-phenylindole (**A′′′–D′′′**). **A′′** to **D′′** are merged images. Scale bars: 10 $\mu$m (**A-B′′′**); 20 $\mu$m (**C–D′′′**).

isoforms in SMA patients. In this study, lymphoblastoid cell lines from controls carrying two *SMN1* genes and none to two *SMN2* genes, and SMA patients lacking *SMN1* gene but carrying two or four *SMN2* genes, were analyzed. SMA patients were born to consanguineous parents and shared common haplotypes at the 5q13 region, indicating that the genomic organization at the *SMN2* locus was identical on both 5q13 alleles.[1] RT-PCR analysis of SMN transcripts revealed, as expected, the absence of SMN$^{\Delta 7}$ transcripts in controls without the *SMN2* gene, and a higher level of SMN$^{\Delta 7}$ in SMA patients (Figure 6). Interestingly, similar levels of full-length and truncated SMN RNAs were observed in controls lacking *SMN2* but carrying two *SMN1* genes and in SMA patients lacking *SMN1* but carrying two *SMN2* genes (Figure 6 and data not shown), respectively. These data indicate that *in vivo* SMN$^{FL}$ and SMN$^{\Delta 7}$ RNAs have similar stability.

Proteins were extracted from cell lines, and immunoblotting experiments were performed using antibodies directed against the N-terminal part of the SMN protein (SMNtl), against exon 7 (hSMNex7-5381), or against SMN$^{\Delta 7}$ (hSMNex8-5699; Figure 6). Importantly, our data did not reveal any signal at the expected size using antiserum against SMN$^{\Delta 7}$ protein. This indicates that the SMN protein detected by SMNtl or hSMNex7-5381 antisera corresponds to the SMN$^{FL}$ form only. Interestingly, this analysis shows a tight correlation between SMN$^{FL}$ protein level and the number of *SMN2* genes in both controls and SMA patients (Figure 6). An increased amount of SMN$^{FL}$ was observed in controls carrying an identical number of *SMN1* genes ($n$ = 2) but an increased number of *SMN2* genes (from 0 to 2). A similar correlation was observed in SMA patients. These data demonstrate the absence of SMN$^{\Delta 7}$ in lymphoblastoid cell lines from controls and SMA patients. The data also reveal a contribution of the SMN$^{FL}$ protein, encoded by the *SMN2* gene, to the total amount of SMN in both controls and SMA patients.

To determine whether the protein profiles in other cell types were similar, immunoblotting experiments of proteins extracted from fibroblasts of controls and SMA born to unrelated parents (Figure 7) were performed. No

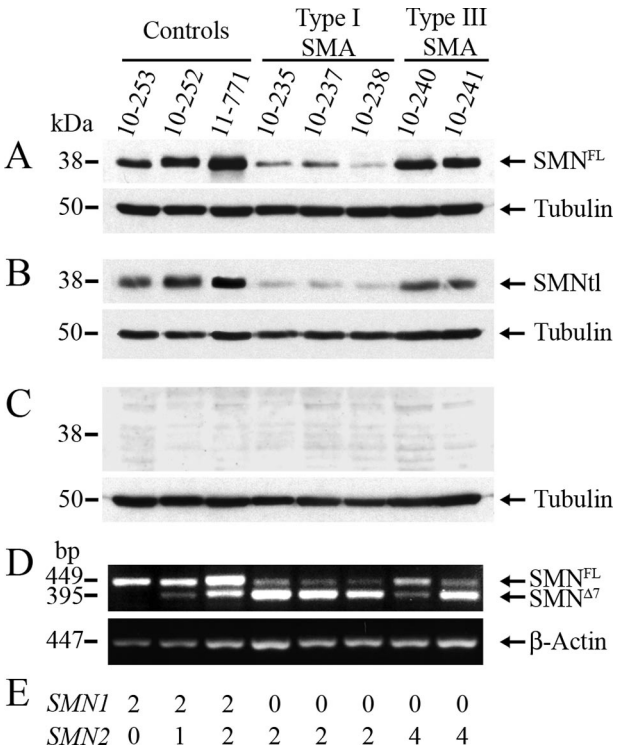


**Figure 6.** Correlation of $SMN^{FL}$ protein level with *SMN2* gene copy number and the absence of $SMN^{\Delta7}$ protein in lymphoblastoid cell lines. Immunoblot analysis of proteins extracted from lymphoblastoid cell lines derived from controls, type I, or type III SMA patients. Immunoblots were incubated with hSMNex7-5381 (**A**), SMNtl monoclonal antibody (**B**), or with hSMNex8-5699 (**C**). Tubulin was used as internal control for loading. **D:** RT-PCR analysis of RNA extracted from the same cell lines, using primers flanking *SMN* exon 7, showing the absence of $SMN^{\Delta7}$ transcripts in the 10-253 cell line and the reduction of $SMN^{FL}$ transcripts in SMA patients. β-Actin was used as internal control. The 10-253 cell line, lacking the *SMN2* gene, was used as a negative control for the detection of $SMN^{\Delta7}$ RNA or protein. **E:** The number of *SMN1* or *SMN2* genes in each control or patient is shown.

$SMN^{\Delta7}$ was detectable either in these cell lines. Although a marked reduction of $SMN^{FL}$ protein was observed in the SMA type I patients, type II or type III patients, carrying the same number of *SMN2* genes, may express very low or high level of $SMN^{FL}$ (Figure 7).

## *Absence of $SMN^{\Delta7}$ Protein and Dramatic Reduction of $SMN^{FL}$ in All Human SMA Tissues Examined*

To determine whether the $SMN^{\Delta7}$ protein exists in human tissues, Western blot analysis was performed on proteins extracted from various tissues from aborted fetuses predicted to have type I SMA or a disease unrelated to SMA (Figure 8). Several tissues were analyzed from both SMA and control, including skeletal muscle, liver, brain, and spinal cord. No $SMN^{\Delta7}$ was detectable using antibody specific to $SMN^{\Delta7}$ (Figure 8). These data indicate that $SMN^{\Delta7}$ is absent, or present at undetectable levels, in SMA tissues.

In addition, a dramatic reduction in $SMN^{FL}$ was observed in SMA samples, regardless of the tissue type examined (Figure 8C). Interestingly, Western blot analysis of SMN did not reveal any difference in the level of

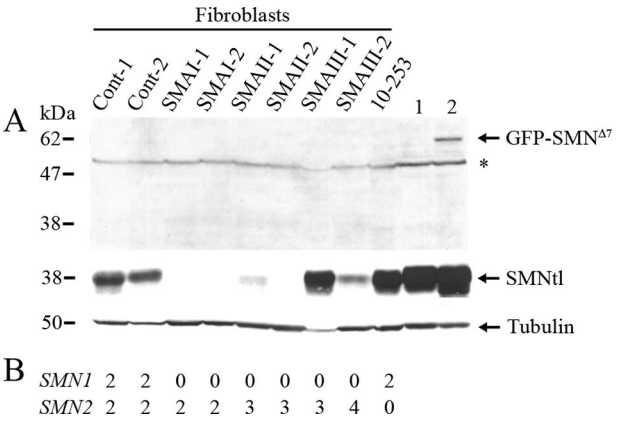


**Figure 7.** Absence of $SMN^{\Delta7}$ protein in fibroblasts of controls and SMA patients. Immunoblot analysis of total protein extracts from fibroblasts derived from controls or type I, II, or III SMA patients. **A:** The membranes were incubated with hSMNex8-5699 (top), SMNtl (middle), or tubulin (bottom). The 10-253 lymphoblastoid cell line was used as a negative control for the $SMN^{\Delta7}$ protein. HeLa cells transfected with the plasmid expressing GFP-$SMN^{\Delta7}$ (**lane 2**) were used as a positive control for the detection of $SMN^{\Delta7}$ protein when compared with GFP only (**lane 1**). **B:** *SMN1* and *SMN2* gene copy number in each control or patient. **Asterisk** indicates a nonspecific band because the 10-253 individual does not carry the *SMN2* gene from which $SMN^{\Delta7}$ transcript is produced.

$SMN^{FL}$ between brain and spinal cord. Thus the vulnerability of the spinal cord in SMA cannot be ascribed to the differential expression or a more dramatic reduction of $SMN^{FL}$ in spinal cord when compared with brain tissue. Moreover, our data show a dramatic reduction in the level of SMN protein early during development.

## *Discussion*

Here, we show that the $SMN^{\Delta7}$ protein is absent or present at very low level in human controls, in SMA tissues, and in cell lines. These results exclude the hypothesis of a dominant effect of $SMN^{\Delta7}$ to explain the vulnerability of motor neurons in SMA. These data are, however, consistent with the absence of symptoms in individuals carrying one or two *SMN1* genes and a varying number of *SMN2* genes. In addition, these data are in agreement with the correlation between the number of *SMN2* genes and the clinical expression of the disease; a high number of *SMN2* genes being associated with the mildest form of SMA.[1,2,27]

The lack of $SMN^{\Delta7}$ protein is attributable to the instability of $SMN^{\Delta7}$ at both RNA and protein levels, depending on the cell type. The instability of $SMN^{\Delta7}$ RNA was strongly suggested after semiquantitative analysis of $SMN^{\Delta7}$ RNA of murine neurons transfected with *GFP-$SMN^{\Delta7}$*. A 48% reduction in $SMN^{\Delta7}$ RNA level was observed when compared with $SMN^{FL}$ RNA levels. In addition to RNA instability, $SMN^{\Delta7}$ protein is unstable when compared with $SMN^{FL}$ in transfected neurons in culture (81% reduction). In contrast, no difference was detected between $SMN^{FL}$ RNA levels in control human lymphoblastoid cell lines carrying two *SMN1* genes but lacking *SMN2*, and the level of $SMN^{\Delta7}$ RNA in SMA patients carrying two *SMN2* genes but no *SMN1*. These data

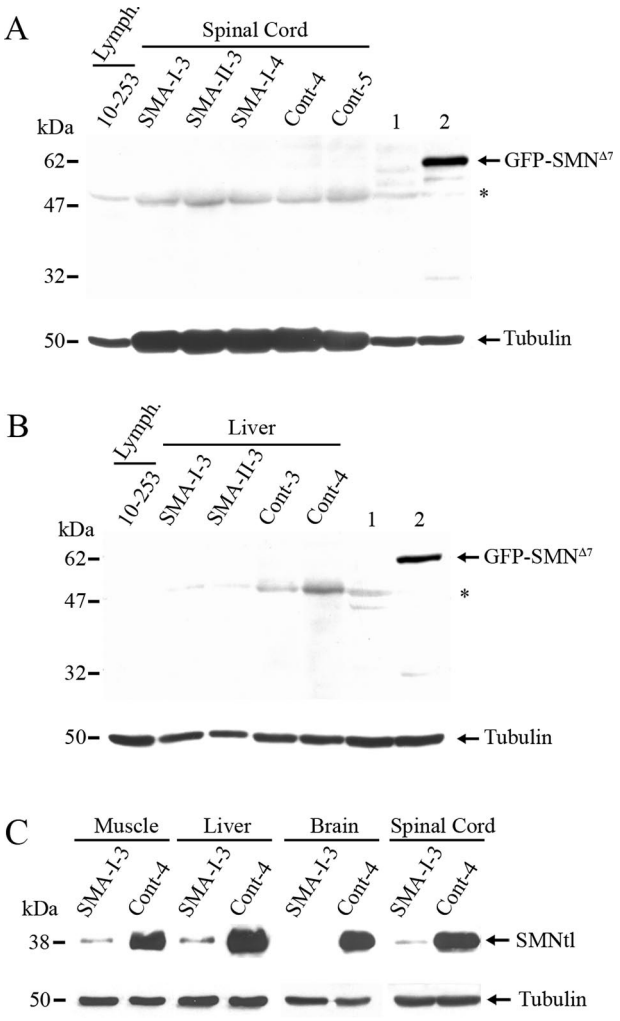


**Figure 8.** Absence of SMN$^{\Delta 7}$ protein and dramatic reduction of SMN$^{FL}$ protein levels in SMA fetuses. Immunoblot analysis of proteins extracted from spinal cord (**A**) or liver (**B**). The membranes were incubated with hSMNex8-5699 antiserum specific to human SMN$^{\Delta 7}$ protein. The 10-253 lymphoblastoid cell line (Lymph.) was used as a negative control and HeLa cells transfected with a plasmid expressing GFP-SMN$^{\Delta 7}$ (**lane 2**) as a positive control when compared with GFP only (**lane 1**). **C:** Immunoblot analysis of proteins extracted from various tissues of the same type I SMA fetus compared with those of control fetus. **Asterisk** indicates a nonspecific band (see comment on Figure 7).

confirm that human SMN$^{\Delta 7}$ and SMN$^{FL}$ RNA have similar stability (P.S.V., unpublished data).[28]

Our study shows that the stability of the SMN$^{\Delta 7}$ protein depends on the cell type. In transfection experiments, SMN$^{\Delta 7}$ protein was detected in HeLa cells and in primary cultures of mouse fibroblasts whereas only very low levels of protein were detectable in primary cultures of mouse neurons. Moreover, the protein stability assay clearly showed a dramatic instability of the GFP-SMN$^{\Delta 7}$ protein in neurons but not in fibroblasts. These data indicate that the stability of SMN$^{\Delta 7}$ is regulated in a cell-dependent manner. The marked instability of SMN$^{\Delta 7}$ protein in neurons but not in other cell types could explain the higher vulnerability of motor neurons in SMA in the case of a putative residual SMN$^{\Delta 7}$ function, although cortical but not spinal cord motor neurons were examined. However, *in vivo*, SMN$^{\Delta 7}$ protein was not detected in either human neuronal (including spinal cord) or nonneuronal tissues. These data indicate that motor neuron degeneration is caused uniquely by a reduction in SMN$^{FL}$ protein levels.

An important question is whether improving SMN$^{\Delta 7}$ protein stability could have a beneficial effect in the treatment of SMA. *In vivo*, homozygous deletions of murine *Smn* exon 7 in all cell types results in embryonic lethality at 9 days postcoitum, whereas *Smn* knockout resulted in earlier embryonic lethality (~3 days postcoitum) suggesting that, *in vivo*, SMN$^{\Delta 7}$ protein might have a residual function.[29,30] These data are further supported by the improved median survival of (*Smn*$^{-/-}$, *SMN2*) mutant mice overexpressing SMN$^{\Delta 7}$.[31] However, SMN$^{\Delta 7}$ is unable to compensate for the complete absence of SMN$^{FL}$ as shown by the severe phenotype of mice carrying a homozygous deletion of *Smn* exon 7 in various cell types.[29,32,33] These data indicate that overexpression of SMN$^{\Delta 7}$ could have a beneficial effect on SMA. In addition to therapeutic strategies aimed at up-regulating *SMN2* gene expression or limiting exon 7 skipping,[34] our study allowed the identification of a new target consisting in improving the stability of SMN$^{\Delta 7}$ protein in SMA patients. These new polyclonal antibodies specific to the SMN$^{\Delta 7}$ protein should be very useful for the detection of drugs able to stabilize this isoform.

It has been hypothesized that the vulnerability of motor neurons in SMA might be related to the differential level of expression of the SMN protein in the spinal cord compared with other tissues. SMN protein analyses in various human controls and SMA fetal tissues presented here do not support this hypothesis. The SMN protein level in SMA fetuses is dramatically reduced at an early stage of spinal cord development, whereas symptoms occur postnatally even in the most severe forms of the disease. These data suggest that during development a protein involved in the same molecular pathway can compensate for the dramatic reduction in SMN. Alternatively, motor neurons may require more SMN protein for axonal outgrowth or maintenance during the postnatal period, which would be in agreement with a link between SMN and axonal transport.

A tight correlation between the amount of SMN protein and the clinical severity of SMA was clearly demonstrated.[3,4] In addition, clinical severity is correlated with the level of SMN$^{FL}$ RNA and the number of *SMN2* genes (P.S.V, unpublished data).[35] Our study shows a tight correlation between the number of *SMN2* genes (from 0 to 4) and SMN$^{FL}$ protein level in both controls and in SMA patients born to consanguineous parents. In these patients, the organization of the *SMN* locus, including the position of the *SMN2* genes on each chromosome, is identical on both alleles. In contrast, in patients born to nonconsanguineous parents, the *SMN2* gene may have a telomeric, centromeric, or an ectopic position on one allele and a different configuration on the other allele. This could explain the weak correlation in SMA patients born to nonconsanguineous parents. Although the sequence of the promoter of *SMN1* and *SMN2* genes is identical,[36,37] *cis*-regulatory elements could be located far from the gene promoter, which, in addition to possible

transacting factors, could regulate expression either at the telomeric locus or at the centromeric locus. The promoter activity of the *SMN2* gene could therefore depend on its genomic environment and thus modulate the response of drugs acting at the level of the gene promoter. A refined analysis of the *SMN2* gene by mapping the 5q13 locus in SMA patients should resolve this issue.

Despite the generation and characterization of several model systems, SMA pathogenesis remains unclear. This is probably attributable to the difficulty in generating model systems that can mimic the situation of human SMA, namely the presence of the *SMN2* locus in its genomic and cellular contexts (motor neurons). Here, we exclude two hypotheses that were proposed from *in vitro* data or transfected cell systems. The generation of novel cellular models, derived from human pluripotent cells able to differentiate into motor neurons, will be very helpful in determining whether motor neuron degeneration in SMA is linked to a defect in metabolism of a subclass of RNA or an additional defective function of SMN unrelated to snRNP biogenesis.

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